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Immunosuppressive property of bromocriptine on human B lymphocyte function *in vitro*

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SUMMARY

Bromocriptine (BRC), a dopamine ergot alkaloid, inhibits the release of pituitary prolactin (PRL). Hypoprolactinaemia induced in rat by treatment with BRC produces a similar immunosuppressive effect as observed in hypophysectomized rats. The effect of immunosuppression by the administration of BRC has been interpreted as the result of hypoprolactinaemia produced by BRC. However, the direct effect of BRC on lymphocyte function has never been evaluated. The purpose of this study was to investigate the *in vitro* effect of BRC on human B cell functions. Highly purified B cells from tonsil samples were isolated by Percoll density gradient from non-rosetted cells, and were used as target cells. BRC significantly suppressed the proliferative response of resting and activated B cells *in vitro*. It suppressed immunoglobulin generation of activated B cells. The inhibition of BRC was manifested in the early stage of the proliferation and differentiation of B cells. The conditioned medium from the polyclonal B cell mitogen-stimulated B cell cultures did not contain PRL as determined by immunoradiometric assay. Treatment with low-dose cyclosporin A or FK506 in conjunction with BRC has proved more effective than either drug alone in suppression of B cell proliferation. Thus, the combined therapy of BRC and immunosuppressants may be effective with decreased toxicity for clinical use.

Keywords bromocriptine B lymphocyte immunosuppression cyclosporin A FK506

INTRODUCTION

Recently, an immunoregulatory role for prolactin (PRL) has been suggested from a number of studies both *in vivo* and *in vitro*. One of the main findings in experimental model to support a link between PRL and the immune system is that hypophysectomized rats have shown depressed immune function, and it may be restored by the administration of PRL [1,2]. The decrease of PRL release by the administration of bromocriptine (BRC) resulted in a similar immunosuppressive effect as observed after hypophysectomy, which is reversed by the administration of PRL. This event is interpreted as the result of inhibition of PRL release by BRC [3–5]. The critical influence of PRL release on maintenance of lymphocyte functions suggests that lymphocytes are an important target tissue for circulating PRL. Actually, antigen-stimulated T lymphocytes express mRNA for PRL in the rat [5]. Concanavalin A (Con A)-stimulated murine lymphocytes synthesize PRL-like molecules [6]. Furthermore, human T lymphocytes, T and B lymphoid cell lines can synthesize biologically active PRL [7,8]. PRL receptors are present on normal lymphocytes and on monocytes in human [9,10]. At suboptimal concentrations of Con A, the addition of rat PRL to murine splenocytes results in an increase in lymphocyte mitogenesis [6]. PRL induces IL-2 receptor on rat splenic lymphocytes *in vitro* [11]. However, in studies designed to evaluate the *in vitro* effects of exogenous PRL on lymphocyte proliferation, the addition of PRL to culture system does not affect proliferative response, although a profound inhibition of cell proliferation results when antisera to PRL are added to neutralize PRL [12]. The mechanism by which PRL interacts with the immune function remains uncertain. Thus, the physiological significance of PRL as an immune receptor regulator is not convincing.

BRC, a dopamine type 2 receptor agonist, prevents the secretion of pituitary PRL. In experimental studies, administration of BRC restored the exacerbation of collagen-induced arthritis [13] or systemic lupus erythematosus (SLE)-like syndrome of autoimmune model mice [14]. BRC decreased early mortality in female NZB/W mice [15], and led to marked decreases in the incidence of experimental autoimmune uveitis and anti-S antigen antibody titres, as well as lymphocyte proliferation assay [16]. In clinical observations, patients with psoriatic arthritis [17] or with SLE [18] showed significant remission after the systemic administration of BRC. These lines of evidence suggest the immunomodulatory effect of BRC. To

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our knowledge, the direct effect of BRC on the lymphocyte function has never been evoked. The aim of this study was to check the direct immunosuppressive effect of BRC on human B lymphocytes.

MATERIALS AND METHODS

Cell preparations

Human tonsils were obtained by tonsilectomy from juvenile patients with chronic tonsillitis, and were dispersed in single-cell suspensions as described previously [19]. Mononuclear cells (MNC) were isolated by Ficoll–Hypaque density gradients. Monocytes and natural killer (NK) cells were depleted by incubation with 5 mM L-leucine methyl ester (Sigma, St Louis, MO) in a serum-free medium. T cells were removed by rosetting twice with 2-aminoethylisothiouronium-bromide (Sigma)-treated sheep erythrocytes (E). In addition, non-rosetted (E^-) cells were purified by isolating the B cells from the interface at 40/50%, 50/60% and 60/70% of a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden). The high-density fraction of B cells from the 60/70% interface, designated as 'resting B cells' [20], were used as target cells unless otherwise indicated.

Reagents

BRC, cyclosporin A (Cs-A) and FK506 were donated by Sandoz (Basle, Switzerland) and Fujisawa Pharmaceutical Co. (Osaka, Japan), respectively. The drugs were dissolved in ethanol, and further diluted in culture medium and added to the cultures. *Staphylococcus aureus* Cowan strain I (SAC), phorbol myristate acetate (PMA) and pokeweed mitogen (PWM) were purchased from Calbiochem Behring Diagnostics (La Jolla, CA), Sigma and GIBCO Laboratories (Grand Island, NY), respectively. Recombinant human IL-2 (rhIL-2) and low molecular weight human B cell growth factor (lmw BCGF) were obtained from Shionogi Pharmaceutical Co., Ltd (Osaka, Japan) and Cellular Products (Buffalo, NY), respectively. MoAbs used in these experiments, FITC-conjugated anti-transferrin receptor (R) antibody (L01.1; anti-CD71), FITC-conjugated anti-CD25 antibody (IL-2R1), anti-CD23 antibody (B6) and anti-HLA-DR antibody (OKIa1) were purchased from Becton Dickinson (Mountain View, CA), Coulter Immunology (Hialeah, FL) and Ortho Diagnostics (Raritan, NJ), respectively.

Measurement of lymphocyte proliferation

Triplicate cultures of lymphocytes were incubated in 200 μl of culture medium with or without stimulators for 3 days at 37°C in 5% CO_2. The culture medium consisted of RPMI 1640 with L-glutamine, supplemented with 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, McLean, VA), streptomycin (100 μg/ml), penicillin G (100 U/ml) and 2-mercaptoethanol (0·005 mM). The cells were pulsed with 1 μCi/well of ^{3}H-thymidine during the last 18 h of culture, unless otherwise stated, and were harvested with a multiple cell harvester.

Cell culture for immunoglobulin generation

Immunoglobulin generation was estimated using a PWM-driven system and an SAC-induced system as described elsewhere [19]. Briefly, in the PWM-driven system, MNC obtained from tonsils were cultured at a cell density of 2×10^5/well for 7 days in the presence of PWM. In the SAC-induced system, high density B cells from tonsillar samples were stimulated with SAC ($1:10^5$ v/v) for 2 days. Viable cells were then collected by Ficoll–Hypaque density gradient and recultured at 2×10^5 cells in 200 μl of culture medium, in the presence or absence of IL-2 (100 U/ml). The amount of IgM and IgG secreted in the culture supernatants was then determined by ELISA.

Immunofluorescence

For the detection of cell surface activation antigens, the cells were examined by direct and indirect immunofluorescence using FITC-conjugated $F(ab)_2$ fragment of rabbit anti-mouse immunoglobulin reagent and purified MoAb as described previously [20]. Cell surface immunofluorescence was analysed by flow cytometry (FACScan, Becton Dickinson), and cell size was measured by forward light angle scatter using flow cytometry.

Measurement of PRL

High density B cells were stimulated with or without SAC in the presence or absence of FCS in culture medium, and each culture supernatant from day 1 to day 3 was collected and kept at −20°C until use for analysis of PRL. Concentrations of human PRL (hPRL) immunoreactivity in conditioned medium were assayed by immunoradiometric assay (IRMA) technique as previously described [21]. MoAb to hPRL and the hPRL standard preparations (first International Reference Preparation (IRP); 650 mU/sample standardized by WHO) were used for the IRMA. The minimal sensitivity of hPRL detection in this method is 0·3 ng/ml.

Cell viability

The viability of B cells cultured in microwells in the presence or absence of BRC for 3 days was examined by trypan blue dye exclusion.

Statistical analysis

The data are represented as the mean ± s.e.m. of experiments. Differences were analysed for significance by Student's *t*-test.

RESULTS

Suppression of the proliferative response of human B lymphocytes by BRC

We examined the effect of BRC on the proliferative response of resting B lymphocytes induced by polyclonal B cell mitogens. High density B cells from tonsillar samples, which were designated as 'resting' B cells, were cultured with SAC or PMA for 3 days. At concentrations of 0·1–100 μg/ml, BRC suppressed DNA synthesis of SAC-stimulated B cells in a dose-dependent manner. BRC also inhibited the proliferative response of PMA-stimulated B cells in the same dose-dependent fashion (Fig. 1a). When B cells were cultured in the presence of different concentrations of SAC, BRC inhibited the proliferative response of B cells to different doses of SAC (data not shown). We next examined the effect of BRC on activated B cells. High density B cells were stimulated with SAC for 3 days, and were then washed thoroughly and recultured in the presence or absence of BRC for an additional 3 days. The SAC-stimulated B cells showed significant levels of spontaneous DNA synthesis without factors in the subsequent 3-day cultures. The presence of rhIL-2 (100 U/ml) or the BCGF preparations (10% v/v) in the

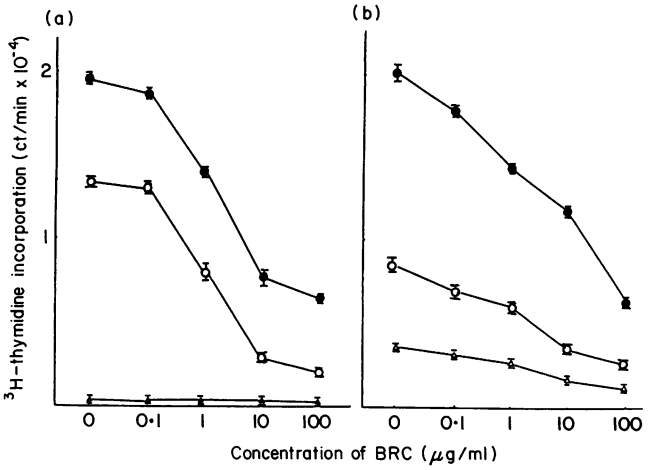

Fig. 1. Effect of bromocriptine (BRC) on resting and activated B lymphocytes. (a) High density B cells from tonsillar samples were cultured for 3 days in the presence of various concentrations of BRC together with *Staphylococcus aureus* Cowan I (SAC) (1:10^5 v/v; ●) or phorbol myristate acetate (PMA) (10 ng/ml; ○). (b) SAC-stimulated B cells were recultured for an additional 3 days in the absence (△) or presence of IL-2 (100 U/ml; ●) or B cell growth factor (BCGF) (10% v/v; ○). Different concentrations of BRC were added at the initiation of the secondary culture. During the last 18 h of culture, ^{3}H-thymidine was added. The results are expressed as mean ± s.e.m. of triplicate cultures. The representative data from three separate experiments are shown.

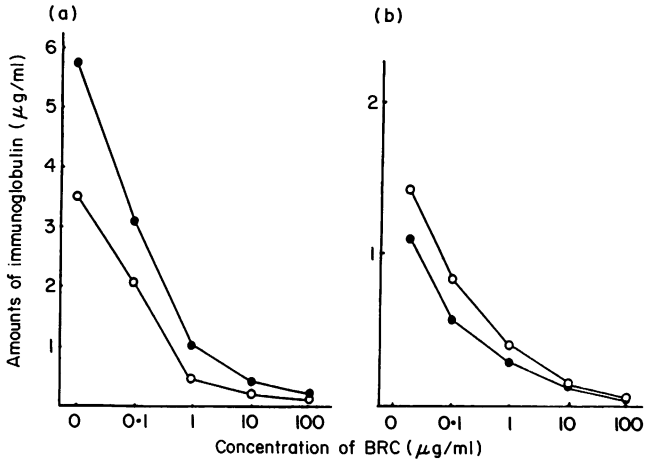

Fig. 2. Effect of bromocriptine (BRC) on immunoglobulin generation by pokeweed-mitogen (PWM)-driven system and *Staphylococcus aureus* Cowan I (SAC)-induced system. (a) Peripheral blood mononuclear cells (PBMC) were cultured in the presence of PWM (1:25 v/v dilution) for 7 days. (b) In SAC-induced system, prestimulated B cells were incubated with IL-2 (100 U/ml) in the presence of BRC for 7 days. The amounts of IgG (●) and IgM (○) secreted in the culture supernatants were measured by ELISA. The results are expressed as mean of triplicate culture supernatants and representative data of five separate experiments are shown.

Table 1. Pretreatment of B cells with bromocriptine

Pretreatment (24 h)	Treatment (72 h)	^{3}H-thymidine uptake (ct/min ± s.e.m.)	*P*
Medium	Medium	149 ± 14	
	SAC	11 047 ± 324	
Bromocriptine	Medium	100 ± 22	
	SAC	2222 ± 69	<0·01

High density B cells were treated with bromocriptine (BRC; 10 μg/ml) for 24 h and then washed thoroughly before culture for another 3 days with or without *Staphylococcus aureus* Cowan I (SAC; 1:10^5 v/v). Eighteen hours before harvesting, the cultures were pulsed with 1 μCi/well of ^{3}H-thymidine. The values represent the mean ± s.e.m. of triplicate cultures. The proliferative response of B cells pre-incubated with BRC for 24 h was compared with that of non-treated B cells. Statistical analyses were performed by Student's *t*-test.

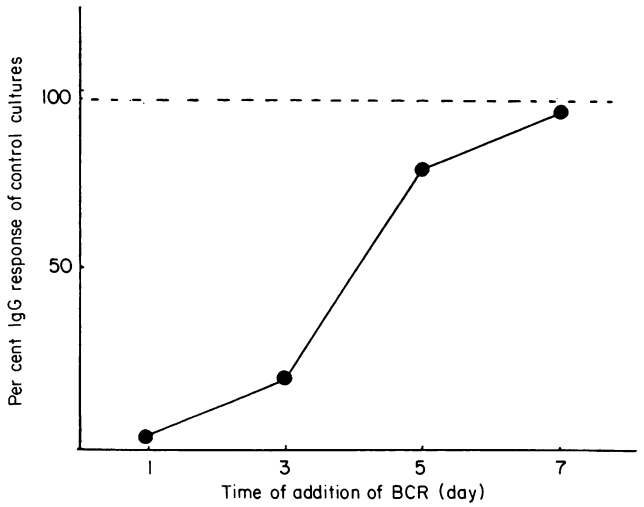

Fig. 3. Kinetics of the effect of bromocriptine (BRC) on immunoglobulin generation by *Staphylococcus aureus* Cowan I (SAC)-activated B cells. Tonsillar B cells were pre-activated with SAC for 2 days, then washed out and re-cultured for another 7 days in the presence of rhIL-2. BRC was added to the cultures at the time indicated. The results are expressed as a percentage of control responses of IgG (1525 ng/ml). Data from two different experiments are shown.

culture enhanced the DNA synthesis of these pre-activated B cells. The addition of BRC markedly inhibited the DNA synthesis of the activated B cells in the presence or absence of growth factors (Fig. 1b). These results indicate that BRC consistently inhibited human B cell proliferation, regardless of their activation state. The dose-response profile showed that 50% inhibitory concentration value of BRC was between 1 and 10 μg/ml, which indicates that the concentration of BRC to induce immunosuppression is rather lower than that used in experimental animal models. We confirmed that at concentrations of 0·1–100 μg/ml, BRC had no cytotoxic effect on human B cells by assessing the B cell viability with trypan blue dye exclusion test.

Kinetic analysis of the effect of BRC on SAC-stimulated B cells

BRC (10 μg/ml) was added at different time intervals after incubation of the B cells with SAC in order to examine when the effect of BRC occurred. BRC exerted inhibitory effect even when it was added at the last 24 h (81%) to similar levels as when added at the first 24 h of culture initiation (83%) during a 72-h culture period (data not shown).

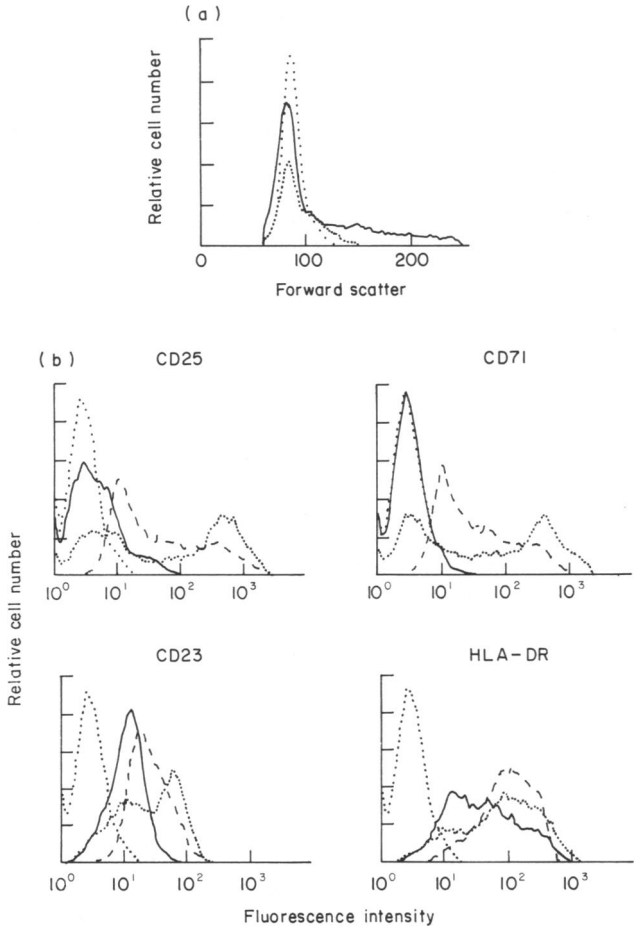

Fig. 4. Flow cytometric analysis of the effect of bromocriptine (BRC) on the expression of activation antigens (b) and cell size (a) of *Staphylococcus aureus* Cowan I (SAC)-stimulated B cells. The percentage of $CD23^+$, $CD25^+$, $CD71^+$ and HLA-DR $(Ia)^+$ cells in each group was as follows; 2·5%, 37·5%, 5·7% and 80·3% in non-stimulated B cells (——), 46·2%, 55·1%, 51·4% and 73·2% in SAC-stimulated B cells (·····), and 41·3%, 51·4%, 45·6% and 94·6% in B cells cultured with SAC plus BCR (– –). The size of B cells stimulated without (· · · · · · ·) or with SAC (——) or SAC plus BRC (·····) was measured by forward light angle scatter. Three different experiments were carried out and a representative result is presented.

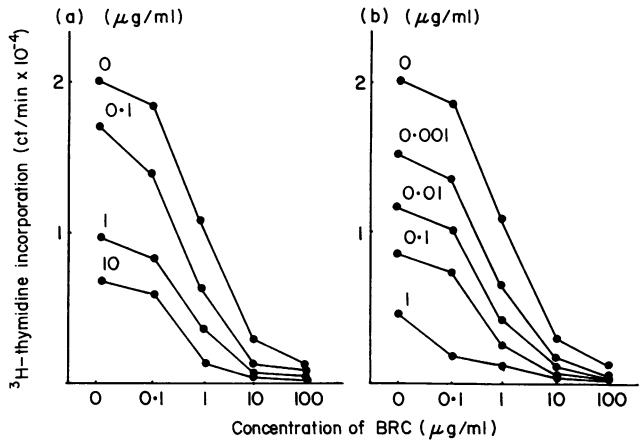

Fig. 5. Effect of addition of different concentrations of bromocriptine (BRC) on cyclosporin A (Cs-A) (a) or FK506 (b) induced immunosuppressive effects. High density B cells were cultured for 3 days with *Staphylococcus aureus* Cowan I (SAC) in the presence or absence of Cs-A (0·1–10 μg/ml) or FK506 (0·001–1 μg/ml). Varying doses of BRC (0·1–100 μg/ml) were added at the initiation of 3-day culture. The values represent the mean of triplicate cultures. Data from two separate experiments are shown.

Effect of pre-incubation of resting B cells with BRC

We examined whether pre-incubation with BRC would have a similar effect on the proliferative response of B cells to SAC stimulation. We incubated high density B cells for 24 h in the presence or absence of BRC (10 μg/ml). These B cells were then washed thoroughly and cell density adjusted to 1×10^5/ml and re-cultured for another 3 days in the presence or absence of SAC. The pre-incubation of resting B cells with BRC demonstrated the decrease of DNA synthesis to subsequent stimulation with SAC (Table 1).

Inhibition of immunoglobulin generation in human B lymphocytes by BRC

To examine the effect of BRC on immunoglobulin synthesis of B cells, tonsillar MNC were cultured with PWM in the presence of BRC for 7 days, and the amounts of immunoglobulin secreted in the culture supernatants were determined by ELISA. The results showed that at concentrations of 0·1–100 μg/ml, BRC suppressed immunoglobulin generation in a dose-dependent manner (Fig. 2). As PWM-driven immunoglobulin generation system depends on T cells and macrophages or monocytes, we further examined the effect of BRC on immunoglobulin production in a T cell-independent system. The B cells were stimulated with SAC for 48 h, then washed throughly and re-cultured in the presence or absence of rhIL-2 for another 7 days. The amounts of immunoglobulin in the culture supernatants were then determined. The presence of rhIL-2 induced significant amounts of immunoglobulin production by B cells in this system. Addition of BRC into the culture at concentrations of 0·1–100 μg/ml resulted in a reduction in immunoglobulin secretion in a similar manner as shown in the PWM-driven system (Fig. 2). These results indicate that the inhibitory action of BRC was directed to B cells, not mediated through T cells or macrophages. As shown in Fig. 2, 50% suppressive concentration value of BRC was between 0·1 and 1 μg/ml, which indicates that the suppressive effect of BRC was more powerful on the anitbody production than on the proliferative response of B cells.

Time course analysis of inhibitory action of BRC in immunoglobulin secretion

Figure 3 shows the inhibitory action of BRC when added at different time intervals over 7 days at the concentration causing marked reduction of immunoglobulin generation (10 μg/ml) in a SAC-induced immunoglobulin generation system. The presence of BRC throughout the 7-day culture showed its maximal inhibitory effect of immunoglobulin generation by activated B cells, and addition of BRC at 2 days after culture initiation had a similar effect. However, BRC was relatively ineffective when added to the culture at day 5 or later. These data imply that BRC

was involved in an early phase of B cell differentiation that was completed within 2 days.

Effect of BRC on the expression of activation antigens

High density B cells were cultured with SAC in the presence of BRC (10 μg/ml). After 72 h of culture, the cells were then stained with anti-CD25, anti-CD71, anti-CD23 and anti-HLA-DR(Ia) MoAbs, and FITC-conjugated second antibody, then analysed by flow cytometry. SAC-stimulated B cells displayed a remarkable increase of the expression of CD23, CD25, CD71 on the cell surfaces (Fig. 4b). The addition of BRC in the cultures decreased the expression of these activation antigens induced by SAC-stimulated B cell surfaces. As these activation antigens are expressed during the G0 to G1 phase of the cell cycle [22], the results suggest that BRC inhibited the G0 to G1 phase transition of the cell cycle. Though resting B cells express HLA-DR(Ia) antigen on the membranes, SAC stimulation enhanced the Ia density on the B cell membrane during the transition of cells from the G0 to G1 phase, then it gradually decreased through the S, G2 and M phase [22]. The presence of BRC in SAC-stimulated cultures did not reduce the increased levels of Ia expression (Fig. 4b). In the same experiments, B cell size was measured by forward light scatter in non-stimulated, SAC-stimulated and SAC plus BRC-treated conditions. The addition of BRC reduced the size of SAC-stimulated B lymphocytes (Fig. 4a), which means a decrease of RNA synthesis, and also suggests that BRC interfered with the G0 to G1 phase transition.

SAC-stimulated human B lymphocytes do not secrete PRL

It has been reported that a human B lymphoblastoid cell line constitutively synthesizes PRL, which is indistinguishable from pituitary human PRL [7]. In this experiment, we examined whether SAC-activated human B lymphocytes can secrete PRL during 3-day cultures. PRL secretion by human B cells was measured over a 3-day period, but the supernatants obtained after 24, 48 and 72 h culture showed no reactivity in the IRMA (data not shown). This suggests that the immunosuppressive effect of BRC on human B cells is not mediated by its inhibitory action on PRL release in this study.

BRC in combination with Cs-A or FK506 amplifies the suppressive effect on B cell proliferation

There are a few reports suggesting that BRC will enhance the immunosuppressive effect of Cs-A [16,23,24]. We examined whether BRC plus Cs-A or FK506 potentiates the immunosuppressive effect on *in vitro* lymphocyte function. High density B cells were cultured with SAC for 3 days in the presence or absence of different concentrations of Cs-A (0·1–10 μg/ml) or FK506 (0·001–1 μg/ml). The presence of Cs-A or FK506 alone in the culture suppressed the proliferative response of B lymphocytes in a dose-dependent fashion. Addition of BRC at concentrations of 0·1–100 μg/ml in each culture amplified the suppressive effect of both immunosuppressive drugs (Fig. 5). At concentrations of 0·1–1 μg/ml, BRC markedly potentiated the immunosuppressive effect of low-dose FK506 (0·001 μg/ml) or Cs-A (0·1 μg/ml). The combination of BRC and Cs-A or FK506 did not affect the viability of human B lymphocytes during 3-day cultures.

DISCUSSION

Previous studies in experimental models and clinical observations [1–5, 13–18] have interpreted hypoprolactinaemia induced by the administration of BRC as leading to an immunosuppressive phenomenon. In addition, recent studies demonstrate that cells from the immune system express the receptors for PRL, and synthesize PRL [6–10]. These lines of evidence have supported the idea that the suppressive action of BRC is mediated via the inhibition of PRL release. However, we consider that BRC may exert direct influence over immunological function by lymphocytes independent of its inhibitory activity on PRL release. A direct effect of BRC on immune cells has never been evoked. We first examined the effect of BRC on B cell activation in the present study. BRC suppressed DNA synthesis of resting as well as activated B cells. Thus, BRC appeared to suppress the proliferative response of human B lymphocytes, regardless of the B cell activation state. The preincubation of resting B cells with BRC demonstrated the decrease of DNA synthesis to subsequent stimulation with SAC. The suppressive effect appears to be irreversible. BRC may interfere with the G0 to G1 phase transition of the B cell cycle, because it decreased the expression of CD25, CD71 and CD23 on the activated B cell surfaces. Interestingly, BRC did not reduce Ia expression on B cell surfaces. Ia expression might play an important role when B cells present antigen to MHC-restricted helper T cells in T–B interaction. Then, this event may indicate that BRC does not affect the role of B cells as antigen-presenting cells.

The effect of BRC on immunoglobulin generation by B cells was examined by using a PWM-induced system (T cell-dependent) and SAC-induced system (T cell-independent). BRC inhibited immunoglobulin production in both systems. These results indicate that BRC has a direct action on human B cell differentiation. SAC-activated B cells can produce immunoglobulin in the presence of T cell factor by receiving the signal to differentiate before the G1 to S phase of cell division [19]. Therefore, the suppressive effect of BRC on B cell differentiation may be caused by its involvement in G1 phase.

As it has been reported that human lymphocytes have an ability to produce PRL [7,8], SAC-activated B lymphocytes may secret PRL in the culture medium during short-time cultures. To make sure, we checked the PRL content of the culture supernatants from SAC-stimulated B lymphocytes, but our samples did not contain PRL. From these results, we conclude that the suppressive effect of BRC on human B cell activation is not linked to its inhibitory effect on PRL secretion.

Although there are a large number of different disorders associated with autoimmunity, hyperactivity of B lymphocytes and deletion of T cell function are known to be involved in the pathogenesis of autoimmune disease. The immunosuppressive efficacy of BRC or a combined treatment with BRC and Cs-A [16,23,24] has been reported in the treatment of autoimmune diseases [13–18]. These observations indicate the *in vivo* immunomodulatory effect of BRC. Our *in vitro* results agree with the clinical effectiveness of BRC, though it remains unknown whether the *in vivo* effect of BRC is induced by suppression of PRL release or not. Meanwhile, we are investigating the effect of BRC on human T lymphocyte function *in vitro*.

BRC has been clinically used for the treatment of hyperprolactinaemia and Parkinson's disease, with minimal side effects.

Our data demonstrate that the concentration to induce immunosuppression of B cells *in vitro* will be attainable in circulating blood when the drug is administered for these treatments in clinics.

Wilner *et al.* [24] demonstrated *in vivo* administration of low-dose Cs-A in conjunction with BRC to be more effective than either drug alone in suppression of rat lymphocyte proliferation. Our data proved this in *in vitro* experiments in humans (Fig. 5), where BRC in combination with low-dose Cs-A or FK506 produced sufficient inhibition of human B cell proliferation. Clinical therapy for immunosuppression is limited by dose-related toxicity, and the combination of BRC with low-dose Cs-A or FK506 may be useful for immunosuppression with decreased toxicity.

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